REMARKS

Claim 1 has been amended. Claims 3, 5, 9, 12-18, 21-22 and 25-43 were previously withdrawn. Following entry of this amendment, claims 1 and 3-43 will be pending in this application.

Claim 1 has been amended to specify that the cytokine-expressing cellular vaccine comprises proliferation-incompetent tumor cells that express human GM-CSF. Support for this amendment may be found throughout the specification, *e.g.*, at page 22, lines 17-25.

None of the amendments introduces any new matter.

THE REJECTIONS

35 U.S.C. §§ 103(a)
Claims 1, 4, 6-8, 10-11, 19-20, and 23-24

Claims 1, 4, 6-8, 10-11, 19-20, and 23-24 are rejected under 35 U.S.C. § 103(a) over Gri *et al.*, "OX40 ligand-transduced tumor cell vaccine synergizes with GM-CSF and requires CD40-Apc signaling to boost the host T cell antitumor response," J. Immunol., 170: 99-106 (2003) ("*Gri*") in view of US Patent Publication 2003/0035790 ("*Chen*"). Specifically, the Examiner states that *Gri* teaches a method of treating colon carcinoma by administering cells of a carcinoma cell line (*i.e.*, bystander cells) transduced to express GM-CSF and OX40 ligand. The Examiner states that *Gri* teaches that OX40 ligand is functionally equivalent with anti-OX40 antibodies. The Examiner states that *Gri* teaches that the GM-CSF-expressing cells are inactivated by irradiation and envisions using the method for human therapy. The Examiner states that

Gri differs from the instant claims in that it does not specifically exemplify administering anti-OX40 antibodies together with GM-CSF-expressing bystander tumor cells. The Examiner states that *Chen* teaches a method of treating cancer by administering a recombinant adenovirus engineered to express GM-CSF, and an anti-OX40 antibody. The Examiner states that *Chen* teaches GM-CSF may be expressed in mammalian cells and discuss a cancer vaccine approach, wherein cancer cells are isolated from patients, transduced in vitro, irradiated, and administered to patients. The Examiner states that *Chen* teaches that these methods can be applied to treating prostate cancer. The Examiner concludes that it would have been obvious to a person of ordinary skill in the art at the time the invention was made to apply the teachings of *Chen* regarding administration of anti-OX40 antibodies to enhance the immune response against GM-CSF-expressing tumor cells to those of *Gri* to arrive at the instantly claimed method. The Examiner states that one of ordinary skill in the art at the time the invention was made would have been motivated to do so, in view of the recognized need to improve cancer immunotherapy, and have a reasonable expectation of success, in view of the specific teachings of the two cited references. Applicants respectfully traverse.

Applicant submits that the Examiner has mischaracterized the two cited references and has provided no basis that it would have been obvious to a person of ordinary skill in the art at the time of the invention to apply the teachings of *Chen* regarding administration of anti-OX40 antibodies to enhance the immune response against GM-CSF-expressing tumor cells to those of *Gri* and arrive at the instantly claimed method.

On the contrary, the disclosure of *Gri* would have taught away from combining anti-OX40 antibodies with GM-CSF-expressing tumor cells. The Examiner asserts that *Gri* teaches that OX40 ligand is functionally equivalent with anti-OX40

antibodies and points to the last paragraph of *Gri* on page 105 as support. However, the Examiner's analysis is incorrect. The last paragraph of *Gri* states, in part, that "[r]ecent adoptive immunotherapy experiments have shown that the coadministration of anti-OX40 Ab reduces the number of transferred T cells required to obtain remission of pulmonary metastasis and intracranial tumors" (citing Kjaergaard *et al.*, "Therapeutic Efficacy of OX-40 Receptor Antibody Depends on Tumor Immunogenicity and Anatomic Site of Tumor Growth," Cancer Research, 60:5514-5521 (2000) ("*Kjaergaard*"), previously submitted as **Exhibit A**). Applicants point to the disclosure of *Kjaergaard* that describes experiments showing that anti-OX40 antibodies do not necessarily and inevitably treat cancers and that the therapeutic efficacy of anti-OX40 antibodies (referred to as OX40 receptor mAb in *Kjaergaard*) was influenced by a number of factors including the tumor burden, the intrinsic immunogenicity of the tumor as well as the histological site of tumor growth. In particular, Applicants submit that *Kjaergaard* describes that "[w]hereas subdermal and intracranial growth of weakly immunogenic MCA 203 and MCA 205 sarcomas and GL261 glioma were susceptible to the mAb treatment, established ***pulmonary MCA 205 metastases were refractory to the same regimen of treatment.*** Furthermore, the mAb administration had ***no impact on the growth of the poorly immunogenic B16/D5 melanoma.***" (emphasis added; Abstract, p. 5514). *Kjaergaard* concludes that the "successful treatment is mAb dose-dependent and effected by the intrinsic immunogenicity of tumors. It is also evident that the response of a particular tumor to the treatment varies and is dependent on the histological location of tumor growth." (see, p. 5517, first full paragraph). Thus, based on *Kjaergaard*, anti-OX40 antibodies are not functionally equivalent to OX40 ligand. Rather, the simple relationship between OX-40 ligand and anti-OX40 antibodies that is presumed by the Examiner from the *Gri* disclosure is contradicted by the results described in *Kjaergaard.* That is, the effect of OX-40 ligand on the treatment of cancer would not have allowed one

of skill in the art to predict the effect of anti-OX-40 antibodies on the treatment of cancer. The disclosure of *Chen* does not remedy the deficiency.

Instead, *Chen* discloses compositions and methods of treating diseases such as cancer by administering one or more compounds that activate one or more cytokine receptors and one or more compounds that activate one or more costimulatory molecules expressed by activated immune cells. In particular, *Chen* discloses injecting MCA26 tumor-bearing mice intratumorally with an adenovirus expressing murine GM-CSF (mGM-CSF) (see, *e.g.*, Example 10). *Chen* also discloses inducing metastatic colon cancer by implanting MCA26 tumor cells into the left lobe of the liver and then subsequently injecting adenovirus expressing mGM-CSF into tumor-bearing mice (see, *e.g.*, Example 11).

In contrast to *Chen*, applicants have amended claim 1 (and therefore, claims dependent therefrom) to specify that the proliferation-incompetent tumor cells express ***human*** GM-CSF. Applicants submit that this feature distinguishes the claimed invention from *Chen*, which illustrates the expression of murine GM-CSF. In fact, applicants submit that human GM-CSF is not active in murine models and cites as support the following reference: Shanafelt *et al.*, "Identification of critical amino acid residues in human and mouse granulocyte-macrophage colony-stimulating factor and their involvement in species specificity," J. Biol. Chem., 266:13804-13810 (1191) ("*Shanafelt*"), a copy of which is submitted herein as **Exhibit B**. According to *Shanafelt*, although both murine and human forms of GM-CSF share a high degree of sequence homology, they are species specific at both the biological and receptor binding levels. In particular, *Shanafelt* describes critical regions and residues in human and murine GM-CSF that are essential for biological activity and that define their absolute species specificity. Thus, one of skill in the art would not be motivated to express ***human***

GM-CSF as recited in the amended claims, in a murine model because of its species specificity.

The Examiner also asserts that *Chen* teaches GM-CSF may be expressed in mammalian cells and points to paragraphs 0212-0214 as support. However, the Examiner's assertion is incorrect. The mention of mammalian cells appears only in paragraph 0213 of *Chen* and states, in part, that "[a] variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to ***mammalian cell systems*** infected with virus (e.g., vaccinia virus, adenovirus, adeno-associated virus (AAV), retrovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA." (emphasis added). Thus, the disclosure at paragraph 0213 of *Chen* suggests that the protein-coding sequence of GM-CSF may be expressed using viruses that infect mammalian cell systems. In fact, *Chen* describes injecting an adenovirus-expressing mGM-CSF (*i.e.*, a virus) directly into tumor-bearing animals (*i.e.*, mammalian cell system) and does not support the Examiner's assertion that *Chen* teaches GM-CSF may be expressed in mammalian cells, let alone, proliferation-incompetent tumor cells.

Moreover, the Examiner's assertion that *Chen* discusses a cancer vaccine approach wherein cancer cells are isolated from patients, transduced in vitro, irradiated, and administered to patients is irrelevant. The claimed invention relates to the administration of a cytokine-expressing cellular vaccine comprising proliferation-incompetent tumor cells that are selected from the group consisting of allogeneic and bystander cells. *Chen* does not teach or suggest this feature of the claimed invention. Instead, the cancer cells described by *Chen* are autologous cells and are not allogeneic and bystander cells, as recited by the claims.

For at least the above reasons, neither *Gri* nor *Chen*, alone or in combination, renders the claimed invention obvious. Accordingly, applicants request that the Examiner withdraw this rejection.

35 USC §101 - Nonstatutory Double Patenting
Claims 1, 4, 6-8, 10, 11, 19-20, 23 and 24

Claims 1, 4, 6-8, 10, 11, 19-20, 23 and 24 are provisionally rejected under the judicially-created doctrine of obviousness-type double patenting as allegedly being unpatentable over claims 1-33 of copending U.S. Application No. 10/404,662.

Applicants request that this provisional rejection be held in abeyance until this application or copending application 10/404,662 is allowed. At that time, applicants will file a Terminal Disclaimer as is appropriate and proper.

CONCLUSION

In view of the foregoing remarks, applicants request that the Examiner favorably reconsider this application and allow the claims pending herein. If the Examiner believes that a telephone conference would expedite allowance of this application, she is invited to telephone the undersigned at any time.

Respectfully submitted,

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3802-090-27 CIP (105576-0033-102)

Applicants	:	Karin Jooss et al.			
Application No.	:	10/807,449	Confirmation No.	:	3354
Filed	:	March 24, 2004			
Examiner	:	Ilia Ouspenski			
For	:	CYTOKINE-EXPRESSING CELLULAR VACCINE COMBINATIONS			

EXHIBIT B

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Identification of Critical Amino Acid Residues in Human and Mouse Granulocyte-Macrophage Colony-stimulating Factor and Their Involvement in Species Specificity*

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Segments critical to the activity of human granulocyte-macrophage colony-stimulating factor (GM-CSF) were identified by scanning deletion analysis and compared with the critical regions previously identified in the homologous mouse GM-CSF protein. Three of the four critical regions thus identified are in equivalent positions in their respective polypeptides, while a fourth critical region of each is uniquely located. To investigate whether unique critical regions are responsible for the observed species specificity of human and mouse GM-CSF, all critical regions were substituted into their opposite homologue. This identified one specific, but different, critical region in each homologue that could not be replaced. Further characterization of the nature of the species specificity of these two proteins was accomplished by the generation of a series of human/mouse GM-CSF hybrids. Each hybrid protein was assayed for specific activity on human- and mouse GM-CSF-dependent cell lines. Significant differences in the specific activity of these hybrids was observed, suggesting that different segments of each molecule interact with their respective receptors. Based on these two approaches, individual amino acids were identified that could provide, at least in part, the interactions between these protein ligands and their respective receptors. These residues are Thr-78 and Met-80 in human GM-CSF and Asp-92, Thr-98, and Asp-102 in mouse GM-CSF.

Granulocyte-macrophage colony stimulating factor (GM-CSF)[1] is a protein hormone that mediates the proliferation, differentiation, and functional activity of neutrophils, macrophages, and eosinophils (2, 3). It is produced by several cell types such as T-lymphocytes and monocytes (4). Both human and mouse GM-CSF have been characterized by cloning, sequencing, and expression of their cDNAs (5, 6). Despite a high level of amino acid homology and similar physical characteristics (7, 8), the two polypeptides are species specific at both the biological and receptor binding levels (6).[2]

Structure-function studies carried out by us and other laboratories (1, 9–12) have identified a number of residues critical to the activity of mouse and human GM-CSF. Most of the results support the four α-helical bundle model for human GM-CSF proposed by Parry *et al.* (13). However, none of these studies suggest a basis for the observed species specificity of the two polypeptides.

In the present report we address this question. Two independent approaches were used. First, regions critical to the activity of human GM-CSF were determined and compared with those previously identified in mouse GM-CSF (1). Second, a series of human/mouse hybrid polypeptides were generated and analyzed. The results of these experiments compliment each other and indicate that different regions of these structurally homologous proteins provide critical contributions to activity, suggesting a basis for the species specificity of biological activity observed with human and mouse GM-CSF.

MATERIALS AND METHODS

Bacterial Host Strains and Vectors—*Escherichia coli* K12 strain JM101 (14) was used as the host for propagation and maintenance of M13 DNA. CJ236 (15) was used to prepare uracil-DNA for use in site-directed mutagenesis. AB1899 (16) was used as the host for expression of wild-type and mutant human and mouse GM-CSF proteins. Either pINIIIompH3 (17) or pOMPTH3 (a tetracycline-resistant variant of pINIIIompH3) was used as the expression vector for all GM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (18). The human GM-CSF coding region was synthetically reconstructed using a series of oligonucleotides. Unique restriction sites were introduced where possible without altering the protein sequence, and preferred *E. coli* codons (19) were selectively used. From this construct, an *Xba*I-*Bam*HI fragment containing the *ompA* leader sequence and the entire human GM-CSF gene was cloned in M13 mp19 (replicative form) and used as the template for site-directed mutagenesis. Construction of the mouse GM-CSF gene and mutagenesis template has been previously described (1).

Mutagenesis, Recombinant DNA, and Sequencing Protocols—*In vivo* recombination of human and mouse GM-CSF was performed as follows: GM-CSF cDNA sequences were oriented in tandem, separated by *Eco*RI and *Sac*I restriction sites, in pINIIIompH3 (17), and were transformed into AB1899. Plasmid DNA was prepared from a bulk culture, cleaved with *Eco*RI and *Sac*I, and retransformed. Individual colonies were screened by restriction enzyme mapping and sequenced using the dideoxynucleotide method (20) with modifications described in the Sequenase™ (United States Biochemical) protocol.

Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (15). M13 (replicative form) DNA containing correct mutations was cleaved with *Xba*I and *Bam*HI (New England Biolabs) for cloning into pINIIIompH3 or cleaved with *Xba*I and *Eco*RI for cloning into pOMPTH3.

Preparation and Quantitation of Protein Extracts—Protein extracts were prepared as previously described (1). Small aliquots (2–10 μl) of each sample were assayed for protein concentration in duplicate using a Schleicher & Schuell Minifold II Slot-Blot system. Purified *E. coli*-derived recombinant human or mouse GM-CSF was used on each

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[1] The abbreviation used is: GM-CSF, granulocyte-macrophage colony-stimulating factor.

[2] A. B. Shanafelt and R. A. Kastelein, unpublished observations.

blot to generate a standard curve for the quantitation of wild-type and mutant protein in each extract. The primary antibody was anti-human GM-CSF peptide hybridoma 2F10 (21) used at a concentration of 1 μg/ml, or anti-mouse GM-CSF peptide hybridoma mg 1.8.2 (22) used at a concentration of 0.2 μg/ml, and secondary antibody was ^{125}I-labeled sheep anti-rat IgG (Amersham Corp.) used at 1:1000 dilution. Autoradiograms were scanned by an LKB Ultroscan XL laser densitometer, and the concentration of wild-type and each mutant GM-CSF protein was calculated from the height of each absorption peak in comparison to that generated from a purified GM-CSF standard curve. The error in the calculated concentration of GM-CSF protein by this method was estimated to be approximately 2-fold based on repetitive samples of individual clones of GM-CSF expressed in AB1899.

Proliferation Assays for Human and Mouse GM-CSF Activity—Human GM-CSF deletion protein extracts were assayed using the human GM-CSF-dependent erythroleukemic cell line TF1 (23). GM-CSF protein extracts with substituted critical regions were assayed on both the TF1 cell line and the mouse GM-CSF-dependent myeloid leukemia cell line NFS60. Sample concentrations were adjusted to 108,000 pg/ml and titrated in quadruplicate to 1.8 pg/ml. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay described by Mosmann (24) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). The concentration of each mutant and wild-type GM-CSF that gave 50% maximum response was determined, and specific relative activity was calculated using the relationship

$$\% \text{ activity} = ([\text{wild-type}]_{1/2}/[\text{mutant}]_{1/2}) \times 100\%,$$

where $[\text{wild-type}]_{1/2}$ and $[\text{mutant}]_{1/2}$ are the concentrations of wild-type and mutant GM-CSF proteins, respectively, that gave 50% maximum response in the TF1 or NFS60 assays. The specific activity determined varied <10% for a given mutant in multiple assays performed over several months time.

RESULTS

Systematic Introduction of Deletions Throughout Human GM-CSF—The structural and/or functional importance of specific regions within the human GM-CSF polypeptide were examined by the systematic introduction of deletions along the entire length of the molecule. In a manner identical to that described for mouse GM-CSF (1), deletions of three amino acids were introduced every five amino acids on a synthetically generated human GM-CSF DNA sequence by site-directed mutagenesis. The amount of wild-type and mutant polypeptide produced was found to vary from 10–100 μg/ml. Samples were assayed in quadruplicate for their ability to stimulate the proliferation of the human GM-CSF-dependent cell line TF1. Results were expressed as specific activity relative to that of native human GM-CSF produced and assayed under the same conditions in parallel. The sensitivity of the assay was estimated to be 0.01% of wild-type activity.

Definition of Four Regions Critical to the Activity of Human GM-CSF—Fig. 1 illustrates the relative biological activity of 56 deletion proteins with respect to the location of their deletions. This representation displays the relative contribution of each segment of the molecule to the activity of intact human GM-CSF, revealing both critical and noncritical regions. Any mutant protein exhibiting less than 0.01% of wild-type activity was considered inactive, and the amino acid residues in the corresponding deletion mutant were referred to as critical to the activity of human GM-CSF. Four critical regions can be identified comprising ≈36% of the residues. The other 64% of the molecule can tolerate small deletions without complete loss of activity. However, it is evident from Fig. 1 that even the small deletions result in substantial loss of activity.

Comparison of Critical Regions in Human and Mouse GM-CSF—Fig. 2 summarizes the locations of critical regions identified in human GM-CSF compared with those previously

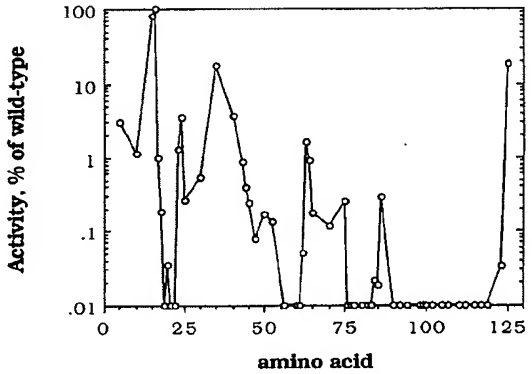

FIG. 1. **Relative activity as a function of deletion location.** The percent activity relative to wild-type human GM-CSF of each mutant protein in the TF1 assay is plotted as a function of the location of the central amino acid of the respective deletion. Note the logarithmic scale used for % activity. The sensitivity of the assay was estimated to be 0.01% of the wild-type activity; any mutant protein with less than this activity was scored as inactive.

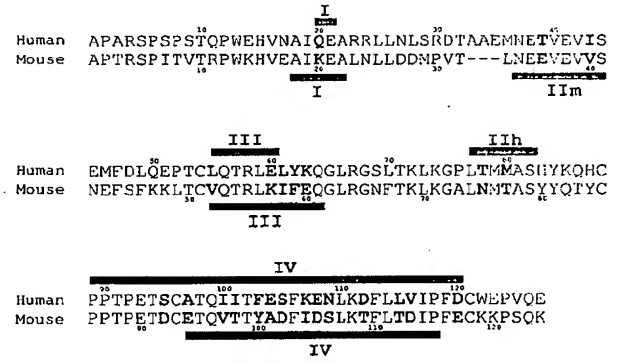

FIG. 2. **Comparison of critical regions between human and mouse GM-CSF.** The amino acid sequences of human and mouse GM-CSF are shown in parallel, with a three-base insertion in the mouse sequence for alignment purposes. Overall, the two molecules are 54% identical. Critical regions are indicated with *bars* above (human) and below (mouse) the respective amino acid sequence. Mismatched amino acids between the two homologues within the critical regions are shown in *boldface type*.

identified in mouse GM-CSF (1). We have adopted a nomenclature for these critical regions in human GM-CSF such that their numerical designation parallels that of those found in mouse GM-CSF (1). It is immediately apparent that the relative positions for critical regions I, III, and IV are comparable in both polypeptides. However, the fourth critical region, labeled IIh in human GM-CSF and IIm in mouse GM-CSF, appears at a unique location in each polypeptide.

Substitution of Critical Regions Has Different Effects on Human and Mouse GM-CSF—The effect of individual critical regions on the activity of human and mouse GM-CSF was examined by substitution of each critical region, including critical regions IIh and IIm, by the corresponding amino acids of the opposite homologue. Substitutions required to change each critical region into the opposite homologue are highlighted in Fig. 2. Mutant proteins were expressed in either pOMPTH3 (human GM-CSF recipient gene) or pINIIIompH3 (mouse GM-CSF recipient gene), and quantitated as described. All mutants were assayed on both the TF1 and NFS60 cell lines, and relative activity to human GM-CSF (in

TABLE I

Activities of GM-CSF proteins with substituted critical regions

Nomenclature: hu, human; mo, mouse; numerals following indicate critical region substituted. hM2, mouse critical region IIm substituted into the corresponding region of human GM-CSF; mH2, the parallel mutant in mouse GM-CSF. Values are expressed as the % activity relative to native human GM-CSF in the TF1 assay or native mouse GM-CSF in the NFS60 assay (see "Materials and Methods").

Mutant[a]	TF1	NFS60
	% activity	
Mouse critical regions in human GM-CSF		
hu1	>100	≤0.01
hu2	**≤0.01**	≤0.01
hu3	11	≤0.01
hu4	19	≤0.01
hM2[a]	>100	≤0.01
hGM-CSF	100	≤0.01
Human critical regions in mouse GM-CSF		
mo1	≤0.01	>100
mo2	≤0.01	>100
mo3	≤0.01	>100
mo4	≤0.01	**0.20**
mH2[a]	≤0.01	>100
mGM-CSF	≤0.01	100

[a] Changes residues not identified as critical in the recipient polypeptide.

the TF1 assay) and mouse GM-CSF (in the NFS60 assay) was determined (Table I).

In human GM-CSF, replacement of critical region I by that of mouse GM-CSF (mutant hu1) had no deleterious effect on activity and substitution of critical regions III and IV had only a moderate effect (mutants hu3 and hu4, respectively) in the TF1 assay. However, substitution of critical region IIh (hu2) resulted in a complete loss of measurable activity (≤0.01%, Table I). In contrast, substitution of critical region IIm (mo2), as well as critical regions I and III (mutants mo1 and mo3, respectively) in mouse GM-CSF by the corresponding sequences in human GM-CSF had no negative effect on activity in the NFS60 assay (Table I), whereas replacement of critical region IV with the corresponding human GM-CSF amino acid residues (mutant mo4) reduced activity to 0.20%.

The inactive human mutant hu2 contains only two amino acid changes, Thr-78 changed to Asn and Met-80 to Thr. Individual substitution of Thr-78 with Asn in human GM-CSF gave a molecule with full activity, while substitution of Met-80 with Thr resulted in a molecule with moderately decreased activity (34%). This demonstrates that individual substitution of these two amino acids in human GM-CSF has little or no effect on activity, but the dual change is sufficient to eliminate the activity of the protein. Mouse GM-CSF mutant mo4 has 14 changes in 26 amino acids, making it difficult to ascribe the loss of activity to any specific amino acid residue. However, analysis of human/mouse GM-CSF hybrid proteins identified mouse residues Asp-92, Thr-98, and Asp-102 as critical for maintaining biological activity (see below).

Substitution of mouse critical region IIm into the corresponding location of human GM-CSF had no negative effect on activity in the TF1 assay, nor did substitution of human critical region IIh into mouse GM-CSF have a negative effect in the NFS60 assay (hM2 and mH2, respectively; Table I). None of the human GM-CSF mutants had any activity in the NFS60 assay, nor did any mouse GM-CSF mutant have any activity in the TF1 assay (Table I).

Generation of Hybrid GM-CSF Polypeptides—A second, independent approach to identify regions and/or residues essential for biological activity uses the species specific character of human and mouse GM-CSF. Hybrid proteins were generated by either *in vivo* recombination or site-directed mutagenesis (15) on appropriate templates. The naming of the hybrid proteins follows the convention that either an H (human) or M (mouse) designates the species of the N-terminal portion of the hybrid, and the following numbers indicate the amino acids at which crossover occurs between the species. For example, H 6/7 contains the first six amino acids of mature human GM-CSF (Ala = 1) and amino acids 7–124 of mature mouse GM-CSF (Ala = 1).

The altered GM-CSF coding regions were expressed and quantitated as described. All mutant polypeptides were assayed on both the TF1 and NFS60 cell lines and relative activity to human GM-CSF (in the TF1 assay) or mouse GM-CSF (in the NFS60 assay) was determined (Table II).

Nonsymmetrical Effects of N- and C-terminal Substitution on Biological Activity—The effects of substitution on the ends of the two GM-CSF proteins are shown graphically in Fig. 3, where the relative activity in the TF1 or NFS60 assay is plotted against the amino acid sequence crossover point of each hybrid.

The effect of N-terminal substitution (Fig. 3*A*) in human GM-CSF is described by N-terminal mouse/C-terminal human hybrids assayed in the TF1 assay, and on mouse GM-CSF by N-terminal human/C-terminal mouse hybrids assayed in the NFS60 assay. In human GM-CSF, 35% can be substituted to yield a fully active molecule (M 43/43, Table II). A further substitution of hybrid M 43/43 by only one amino acid (M 45/45, additional change of human Ile-43 to Phe) causes a 500-fold decrease in activity. Mouse GM-CSF, on the other hand, loses activity after substitution of only 17 N-terminal amino acids. The single additional amino acid change in the N terminus of Glu-17 to Asn between H 16/17 and H 19/20 in mouse GM-CSF resulted in a 5,000-fold decrease in activity (Table II).

A direct comparison of C-terminal substitutions is shown in Fig. 3*B*. Here, N-terminal human/C-terminal mouse hybrids in the TF1 assay describe C-terminal substitution of human GM-CSF, and N-terminal mouse/C-terminal human hybrids in the NFS60 assay show the effects of C-terminal substitution in mouse GM-CSF. Human GM-CSF loses about 99% of its activity after substitution of only four amino acids (H 121/119, 0.79%); yet in mouse GM-CSF, the C terminus is unaffected by substitution of the terminal 22 amino acids. Not until the additional Asp-102 to Ser change occurs between M 103/107 and M 101/105 is a loss of activity observed. Two other similar activity changes are seen in this region resulting from net single amino acid changes: Thr-98 to Ile (M 97/101) and Asp-92 to Ser (M 91/95).

Single Amino Acid Substitutions of GM-CSF—In a number of cases large changes in activity were observed between hybrids that only differ by one amino acid. To examine these potentially important amino acid positions independent of the hybrid context single amino acid substitution of wild-type human and mouse GM-CSF were made.

Human GM-CSF—Large changes in activity of adjacent hybrids occur at two places, between M 43/43 and M 45/45, and H 122/120 and H 121/119 (Table II). The net amino acid changes were, respectively, Ile-43 to Phe and Trp-122 to Lys. These individual substitutions were generated by site-directed mutagenesis and assayed in the TF1 assay.

The results are shown in Table III, where the activity of each single amino acid mutant is shown in boldface type adjacent to the activity of its corresponding hybrid. Changes

TABLE II

GM-CSF hybrid proteins and their activity on human and mouse cell lines

Hybrid[a]	% of parent polypeptide[b]	Activity, % of wild-type[c]	
		TF1	NFS60
H 6/7[d]	4.7	<0.01	180
H 16/17[d]	12.6	<0.01	50.0
H 19/20	15.0	<0.01	<0.01
H 22/23	17.3	<0.01	<0.01
H 35/36	27.6	<0.01	<0.01
H 44/46	34.6	<0.01	<0.01
H 49/50	38.6	<0.01	<0.01
H 54/52	42.5	<0.01	<0.01
H 59/57[d]	46.5	<0.01	<0.01
H 68/66[d]	53.5	0.08	<0.01
H 75/73[d]	59.1	0.06	<0.01
H 77/75[d]	60.6	0.04	<0.01
H 94/92[d]	74.0	0.17	<0.01
H 119/117[d]	93.7	1.2	<0.01
H 121/119	95.3	0.79	<0.01
H 122/120	96.1	8.6	<0.01
H 124/122	97.6	40	<0.01
H 126/124	99.2	73	<0.01
hGM-CSF	**100.0**	**100**	**<0.01**
M 6/7[d]	4.8	5.6	<0.01
M 22/23	17.7	12	<0.01
M 35/36	28.2	43	0.38
M 43/43[d]	34.7	210	0.13
M 45/45	36.3	0.38	<0.01
M 46/46	37.1	0.18	<0.01
M 47/47	37.9	0.04	<0.01
M 48/48	38.7	1.1	1.3
M 49/50	39.5	0.06	0.64
M 51/55	41.1	0.17	6.6
M 56/60[d]	45.2	0.06	0.29
M 65/69[d]	52.4	<0.01	0.04
M 72/76[d]	58.1	<0.01	0.03
M 74/78[d]	59.7	<0.01	0.04
M 81/85[d]	65.3	<0.01	0.03
M 91/95[d]	73.4	<0.01	0.03
M 93/97	75.0	<0.01	0.90
M 96/100	77.4	<0.01	1.0
M 97/101	78.2	<0.01	1.4
M 99/103	79.8	<0.01	14
M 100/104	80.6	<0.01	17
M 101/105	81.5	<0.01	6.6
M 103/107	83.1	<0.01	110
M 116/120	93.5	<0.01	100
mGM-CSF	**100.0**	**<0.01**	**100**

[a] The nomenclature follows the convention that either an H (human) or M (mouse) designates the species of the N-terminal portion of the hybrid protein, and the following numbers indicate the amino acids at which crossover occurs between the species.

[b] The parent polypeptide is defined as that comprising the N-terminal of the hybrid. The percent composition reflects the number of amino acid residues of the N-terminal species contained in the hybrid divided by the total number of residues in the mature wild-type GM-CSF protein.

[c] Activity is expressed as the % activity relative to native GM-CSF in the TF1 or NFS60 assay (see "Materials and Methods").

[d] These hybrids were generated by *in vivo* recombination as described in "Materials and Methods." All others were made using site-directed mutagenesis on appropriate templates (15).

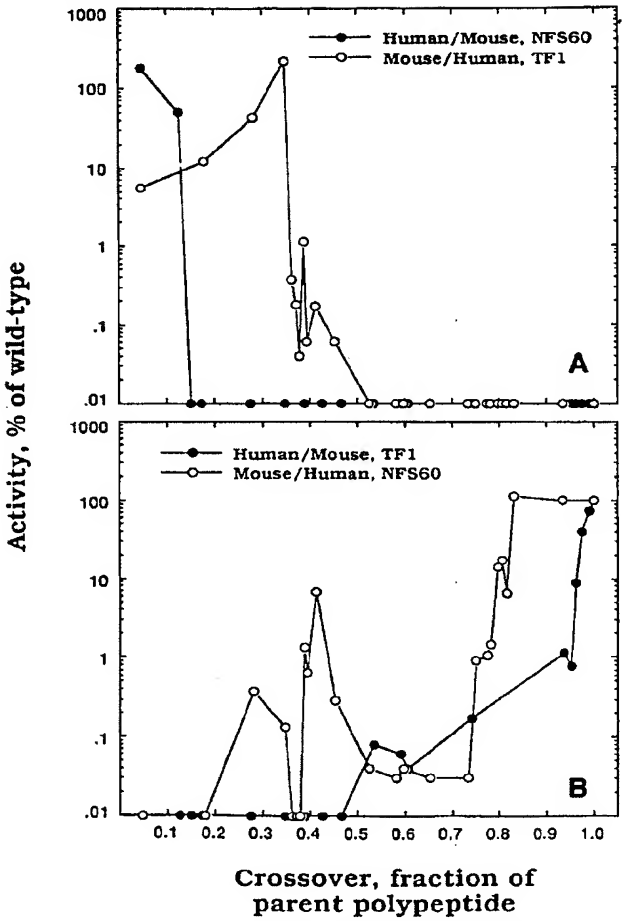

FIG. 3. **Differences in the N- and C-terminal responses between human and mouse GM-CSF.** Activity of the hybrid proteins were measured in the TF1 (human) or NFS60 (mouse) assays. Activity is presented as a function of the fraction of the hybrid that is made up of the N-terminal homologue. Human/mouse hybrids (●) and mouse/human hybrids (○) are grouped cross-assay to compare parallel hybrids. In *panel A*, effects of substitution on activity of the N-terminal regions of both proteins are shown. *Panel B* displays the effects of C-terminal substitution. In both cases, large variations in the response pattern between the two polypeptides are evident.

to Ile-43 and Trp-122 yield proteins with activities similar to the appropriate hybrid, suggesting that Ile-43 and Trp-122 are integral in their contribution to the activity of human GM-CSF.

Mouse GM-CSF—The greatest variations in activity occur between hybrids H 16/17 and H 19/20, M 48/48 and M 47/47, and at the C terminus between M 103/107 and M 101/105, M 99/103, and M 97/101, and M 93/97 and M 91/95, for a combined decrease from 113 to 0.03% activity (Table II). The net amino acid changes were, respectively, Glu-17 to Gln, Lys-48 to Asp, Asp-92 to Ser, Thr-98 to Ile, and Asp-102 to Ser. The activities of the mutants were measured in the NFS60 assay and are displayed in boldface type next to the activity of the corresponding hybrid in Table III.

Both mE17N and mK48D have greater than full activity, indicating that a combined effect of the hybrid substitutions was responsible for the loss in activity in the corresponding hybrid molecules. The single change in mD92S has no significant effect on activity, while mT98I and mD102S both give about a 10-fold reduction in activity. Since these 3 residues fall at or in the only critical region in mouse GM-CSF that was sensitive to substitution (critical region IV) we examined the combined effect of these three mutations on the activity of mouse GM-CSF. Two double mutants, mDT:SI (Asp-92 and Thr-98 to Ser and Ile) and mTD:IS(Thr-98 and Asp-102 to Ile and Ser) still had about 10% activity (Table III).

TABLE III
Comparison of individual amino acid mutants with corresponding hybrid GM-CSF protein

Mutant GM-CSF	Corresponding hybrid	Activities, % of wild-type
Human GM-CSF[a]		
hI43F	M 45/45	**0.50**/0.38
hW122K	H 121/119	**1.3**/0.79
Mouse GM-CSF[b]		
mE17N	H 19/20	**340**/0.01
mK48D	M 47/47	**580**/0.01
mD92S	M 91/95	**66**/0.03
mT98I	M 97/101	**8.5**/1.4
mD102S	M 101/105	**9.2**/6.6
mDT:SI[c]	M 91/95	**12**/0.03
mTD:IS[d]	M 97/101	**5.5**/1.4
mDTD:SIS[e]	M 91/95	**0.02**/0.03

[a] Activities measured in the TF1 assay.
[b] Activities measured in the NSF60 assay.
[c] mDT:SI ≡ D92S + T98I in mouse GM-CSF.
[d] mTD:IS ≡ T98I + D102S in mouse GM-CSF.
[e] mDTD:SIS ≡ D92S + T98I + D102S in mouse GM-CSF.

However, the combined change of all 3 residues produces a molecule with minimal activity (mDTD:SIS, Table III). This triple mutant has activity equal to its corresponding hybrid (M 91/95), indicating that these 3 amino acid residues together act significantly in the activity of mouse GM-CSF. Substitution of the corresponding amino acid residues in human GM-CSF, Ser-95, Ile-101, and Ser-105 to their mouse GM-CSF counterparts had no effect on activity in the TF1 assay.

DISCUSSION

A striking feature of human and mouse GM-CSF is their absolute species specificity despite a high degree of sequence homology. Both molecules are physically similar polypeptides (8) with identical disulfide structures (7), and both are suggested to have comparable structures using predictive algorithms (13, 25). The considerable homology between the two polypeptides suggests that differences exposed by similar forms of analysis are contributing factors to the uniqueness of each molecule. Two independent approaches were used to test this hypothesis. First, we determined and compared regions critical to the biological activity of human GM-CSF with those previously identified in mouse GM-CSF (1). Second, we have generated and analyzed a series of human/mouse hybrid proteins.

Fig. 2 graphically compares the critical regions found in human and mouse GM-CSF. The structural homology between the two polypeptides is reflected in the corresponding locations of three out of the four critical regions (regions *I*, *III*, and *IV*). Only critical regions IIh in human GM-CSF and IIm in mouse GM-CSF occupy unique positions in their respective polypeptides. We anticipated that the difference in location of these critical regions could account (at least to some extent) for the species specific character of GM-CSF. To test this hypothesis we substituted not only the uniquely located critical regions IIh and IIm, but also the common critical regions in both molecules with residues of the opposite species. Both molecules are generally tolerant to substitution with minor or no decrease in activity (Table I). However, in each molecule one specific, but different, critical region is sensitive to substitution. In human GM-CSF, substitution of critical region IIh eliminates activity, while a similar decrease was observed when critical region IV was substituted in mouse GM-CSF.

Critical Amino Acid Residues in Human GM-CSF—Sensitivity of human critical region IIh to substitution with mouse residues supports the hypothesis that the residues comprising this region, amino acids at positions 77–82, contribute to the species-specific character of GM-CSF. The present study identifies specifically residues Thr-78 and Met-80 in this region as important residues. Although individual substitution of these residues does not affect the activity significantly, substitution of both amino acids to the corresponding mouse residues (mutant hu2) completely eliminates it. This double mutant also is unable to compete native human GM-CSF in binding assays on TF1 cells (data not shown).

Two other lines of evidence support the notion that residues 77–82 form species-specific receptor contacts. Brown *et al.* (11) have mapped the epitopes of a pair of blocking monoclonal antibodies to residues 77–94. Secondly, in a previously published study involving hybrid GM-CSF molecules, Kaushansky and co-workers (10) identified residues 78–94 as critical for functional activity. Our hybrid GM-CSF data do not specifically identify human GM-CSF residues 77–82 as critical. An explanation for this could be the low activity of the two hybrids that cover this region (H 77/75 and H 94/92, Table II).

Kaushansky *et al.* (10) also stipulate that amino acid residues 21–31 are critical for activity of human GM-CSF. Our data do not support this conclusion. Substitution of the first 43 residues of human GM-CSF resulted in a protein with full activity (Table II, M 43/43). This indicates that human GM-CSF can sustain extensive substitution in this region, although it is intolerant to small deletions (Fig. 1) and specific substitutions (*e.g.* residues 20 and 21, Ref. 26). Apparently, the structure of human GM-CSF can be disrupted by mutations in the N-terminal 43 amino acids, but it can be maintained by complete substitution with mouse residues. Consequently, this region cannot be involved in the species specificity observed for human GM-CSF.

In the hybrid analysis we observed a sharp reduction in activity across human GM-CSF residues 122–126. This decrease in activity was not observed by either Clark-Lewis *et al.* (9), using synthesized human GM-CSF peptides, or by Kaushansky *et al.* (10). However, neither Clark-Lewis *et al.* nor Kaushansky *et al.* accurately quantitated the amount of specific mutant protein examined in their assays, and thus may explain why these two groups did not see this effect. Deletions in the C-terminal portion of human GM-CSF also caused significant losses of activity (Fig. 1). We have purified to near homogeneity, quantitated, and assayed C-terminal deletion mutants of both human and mouse GM-CSF which lack the final 6 amino acid residues of each protein (data not shown). Consistent with our hybrid data, the human deletion mutant has 3.0% of wild-type activity (compare with H 121/119, 0.79%, and hW122K, 1.3% activity), and the mouse deletion mutant has 68% of wild-type activity (compare with M 116/120, 100% activity). The sensitivity of these residues in human GM-CSF to both substitution (specifically Trp-122) and deletion alludes to the possibility that they have a material role in the activity of this protein, whereas this region appears to be of no importance in mouse GM-CSF.

A specific role for the C terminus of human GM-CSF is further supported by the work of Seelig *et al.* (12). These authors show that purified rabbit IgG raised against a C-terminal peptide of human GM-CSF (residues 110–127) blocks the activity of human GM-CSF. Anti-idiotypic antibodies generated against these IgG proteins did not bind to human GM-CSF or the peptide, yet specifically blocked human GM-CSF activity and inhibited ^{125}I-human GM-CSF

receptor binding. These data support the conclusion that a portion of these residues is close to or in direct contact with the receptor.

Two additional amino acid residues in human GM-CSF were identified as critical for activity in the native polypeptide, Ile-43 and Trp-122, with Trp-122 likely to be at or near the receptor-binding site. At present we have no data to suggest that Ile-43 has a functional role. This residue may be important for maintaining structural integrity.

Critical Amino Acid Residues in Mouse GM-CSF—Tolerance of the unique mouse critical region IIm to substitution with human residues indicates that these residues do not contribute to the species-specific character of mouse GM-CSF. This is true for all cricital regions in human and mouse GM-CSF that retain significant activity upon substitution with residues of the other species. It is likely that these regions are critical (as defined by deletion analysis) because they fulfill a structural requirement of the molecule. Since mouse, but not human, critical region IV is sensitive to substitution, it suggests that this region provides some species-specific feature to mouse GM-CSF. The analysis of hybrid mouse/human GM-CSF molecules across this region identifies 3 residues (Asp-92, Thr-98, and Asp-102) as significant for mouse GM-CSF activity. Just as was observed for residues Thr-78 and Met-80 of the unique human GM-CSF critical region IIh, individual substitution of the 3 mouse GM-CSF residues had no dramatic effect on activity. Substitution of all 3 residues by the corresponding human GM-CSF residues rendered the resulting mutant mouse GM-CSF polypeptide virtually inactive, suggesting that some cooperative effect between these 3 residues is occurring. This effect is unique to mouse GM-CSF, since neither substitution of human GM-CSF critical region IV nor simultaneous substitution of the comparable human GM-CSF residues (residues Ser-95, Ile-101, and Ser-105) had any profound effect on activity.

In mouse GM-CSF, two additional large changes in activity (>100-fold) occurred as an apparent consequence of single amino acid substitutions. However, individual substitution of these residues, Glu-17 and Lys-48, had no deleterious effect on activity, reflecting that some cumulative effect of substitutions in the corresponding hybrid proteins was necessary to affect activity.

What Defines Species Specificity in Human and Mouse GM-CSF?—By analyzing human and mouse GM-CSF using similar approaches, significant differences were exposed despite the overall physical similarities of both molecules. First of all, by substituting critical regions it was found that each molecule contains one specific, but different, critical region that is sensitive to substitution. The unique character of each polypeptide was further reflected by their differential sensitivity to N- and C-terminal substitution with residues of the other species. Thus, the interactions of the two ligands with their respective receptors appear to involve different regions of the ligands. For human GM-CSF, the evidence available suggests that residues in critical region IIh, in particular Thr-78 and Met-80, provide receptor contacts. The C terminus of human GM-CSF, and specifically residue Trp-122, is also either part of or in close proximity to the human GM-CSF receptor-binding domain. In mouse GM-CSF, it is possible that a combination of residues Asp-92, Thr-98, and Asp-102 provides species-specific receptor contacts. A second important region in mouse GM-CSF is located around residues 17–22. At present, no data are available related to whether these residues form receptor interactions or whether they fulfill a critical structural role; however, deletion mutants and N-terminal human/C-terminal mouse GM-CSF hybrids have identified

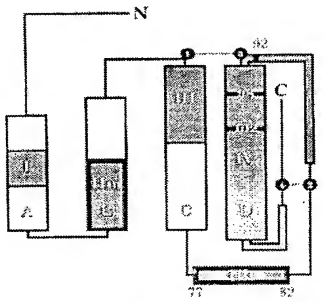

FIG. 4. **Critical regions superimposed on proposed secondary structure of human and mouse GM-CSF.** Secondary structure of the proposed four helix bundel model for GM-CSF is adapted from Parry *et al.* (13), and is drawn approximately to scale. Alphabetically labeled helices are indicated by *rectangular boxes*. Critical regions, indicated by *roman numerals*, are superimposed as *shaded areas* along the linear sequence with the unique critical regions IIh and IIm heavily *outlined* (some superposition of critical regions occurs, *e.g.* IV). Cysteines are numbered sequentially and indicated by *black circles*; disulfide bridges are shown by *dotted lines*. The parallel nature of helices C and D as required by the disulfide pairing is also represented. Mouse GM-CSF residues Asp-92, Thr-98, and Asp-102 are indicated by *heavy lines*.

this region as absolutely required for mouse GM-CSF activity. The full activity of human GM-CSF hybrids with a mouse GM-CSF N terminus of up to 43 residues excludes a contribution of this region to specificity of human GM-CSF.

The differences between human and mouse GM-CSF described above may form the basis for the species specificity observed between these two molecules. We have superimposed these data on a four α-helical bundle model for human GM-CSF proposed by Parry *et al.* (13) (Fig. 4). Strikingly, the shared critical regions between human and mouse GM-CSF, namely I, III, and IV, coincide precisely with regions predicted to be α-helical. This similarity between human and mouse GM-CSF suggests that these helices play an important role in maintaining protein integrity and bioactivity. In human GM-CSF, critical region IIh forms the beginning of the C-D loop. If loops and coils at the end of helices are the most likely sites of receptor interactions (as proposed by Parry *et al.* (13)), it is possible that the residues defining critical region IIh in human GM-CSF form direct receptor contacts; both deletion and substitution eliminate activity. The C-terminal residues 122–127 are potentially in close proximity to the residues of critical region IIh and could form part of the receptor-binding domain. The critical mouse GM-CSF residues Asp-92, Thr-98, and Asp-102 are located in the N-terminal portion of mouse critical region IV, coinciding with helix D in the Parry model (Fig. 4). Structural studies of mouse GM-CSF (27) using proline substitutions suggest that the N-terminal portion of critical region IV, predicted to be α-helical, is only loosely defined by this structural motif. Thus, it is possible that the N-terminal portion of critical region IV, and specifically residues Asp-92, Thr-98, and Asp-102, form a loop-like structure that is free to interact with the mouse GM-CSF receptor.

If the Parry *et al.* (13) model is correct, critical region IIh and the N-terminal portion of critical region IV lie on opposite poles of the molecule (Fig. 4). Indeed, if these are the sites of direct receptor interactions for their respective proteins, the species specificity of human and mouse GM-CSF can be explained on this basis alone. Confirmation of this conclusion is dependent upon further mutational analysis of the residues involved and, eventually, three-dimensional structural information on each polypeptide.

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